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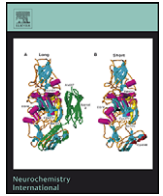
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Effects of Vinpocetine on mitochondrial function and neuroprotection in primary cortical neurons

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ABSTRACT

Vinpocetine (ethyl apovincamine), a synthetic derivative of the Vinca minor alkaloid vincamine, is widely used for the treatment of cerebrovascular-related diseases. One of the proposed mechanisms underlying its action is to protect against the cytotoxic effects of glutamate overexposure. Glutamate excitotoxicity leads to the dysregulation of mitochondrial function and neuronal metabolism. As Vinpocetine has a binding affinity to the peripheral-type benzodiazepine receptor (PBR) involved in the mitochondrial transition pore complex, we investigated whether neuroprotection can be at least partially due to Vinpocetine's effects on PBRs.

Neuroprotective effects of PK11195 and Ro5-4864, two drugs with selective and high affinity to PBR, were compared to Vinpocetine in glutamate excitotoxicity assays on primary cortical neuronal cultures. Vinpocetine exerted a neuroprotective action in a 1–50 μ M concentration range while PK11195 and Ro5-4864 were only slightly neuroprotective, especially in high (>25 μ M) concentrations. Combined pretreatment of neuronal cultures with Vinpocetine and PK11195 or Ro5-4864 showed increased neuroprotection in a dose-dependent manner, indicating that the different drugs may have different targets. To test this hypothesis, mitochondrial membrane potential (MMP) of cultured neurons was measured by flow cytometry. 25 μ M Vinpocetine reduced the decrease of mitochondrial inner membrane potential induced by glutamate exposure, but Ro5-4864 in itself was found to be more potent to block glutamate-evoked changes in MMP. Combination of Ro5-4864 and Vinpocetine treatment was found to be even more effective.

In summary, the present results indicate that the neuroprotective action of vinpocetine in culture can not be explained by its effect on neuronal PBRs alone and that additional drug targets are involved.

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1. Introduction

A longer than transient deficit in focal cerebral perfusion can result in ischemic stroke, which is one of the main causes of mortality and disability in Western societies. A consequence of ischemia is cerebral damage caused mostly by glutamate-

mediated excitotoxicity (for review, see Won et al., 2002). Neuronal release of glutamate following cerebral ischemia causes an excess and sustained activation of glutamate receptors. A massive Na^+ and Ca^{2+} influx through activated glutamate receptors results in increased intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and, as a consequence, in cell swelling. If ATP-dependent Ca^{2+} efflux and/or Ca^{2+} uptake of mitochondria, endoplasmic reticulum and core membrane are impaired, accumulation of Ca^{2+} in the cytoplasm leads to cytotoxicity. Additionally, high $[\text{Ca}^{2+}]_i$ activates Ca^{2+} -dependent enzymes (proteases, kinases, lipases, endonucleases and other catabolic enzymes) and leads to the formation of NO or reactive oxidative species resulting in oxidative stress (Kristian and Siesjö, 1998). These processes eventually cause cell death either due to impairment of membrane structure, cytoskeleton and cellular metabolism or

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Abbreviations: MMP, mitochondrial membrane potential; MPTP, mitochondrial permeability transition pore; PBR, peripheral-type benzodiazepine receptor/translocator protein (18 kDa); VDSC, voltage-gated sodium channel; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} level.

indirectly, via the initiation of apoptotic pathways (Won et al., 2002; Hazell, 2007).

The onset of deleterious cellular events upon increased $[Ca^{2+}]_i$ depends greatly on mitochondrial activity, as the transport of Ca^{2+} from the cytoplasm into the mitochondrial matrix is driven by mitochondrial membrane potential (MMP) (Duchen, 2000; Nicholls and Ward, 2000). MMP is generated in the inner mitochondrial membrane due to a proton gradient provoked by the operation of the respiratory chain. In the depolarised mitochondria, oxidative phosphorylation is hampered and diminished ATP production enhances the cytotoxic effects of Ca^{2+} by increasing $[Ca^{2+}]_i$ (Nicholls, 2004). When mitochondrial Ca^{2+} accumulation persists over longer periods of time, overloaded mitochondria produce oxidative species (Giulivi et al., 2006). As a consequence, mitochondrial permeability transition pore complex (MPTP) is opened, leading to the dissipation of MMP and the stream of accumulated Ca^{2+} into the cytoplasm (Green and Reed, 1998; Nicholls and Ward, 2000). Opened MPTP can also lead to the release of several other cytotoxic compounds (e.g. apoptosis-inducing factor and cytochrome c), which further enhances apoptotic processes (Kroemer and Reed, 2000; Luetjens et al., 2000). Therefore, attempts to preserve neuronal mitochondrial functions under excitotoxic conditions are considered as important strategic approaches (Norenberg and Rao, 2007; Won et al., 2002).

Peripheral-type benzodiazepine receptor (PBR) or as recently renamed, translocator protein 18 kDa (TSPO), is an outer mitochondrial transmembrane protein. Recently, PBR has been regarded as one of the feasible targets to preserve the functions of mitochondria (Casellas et al., 2002; Castedo et al., 2002; Veenman and Gavish, 2006). Namely, PBR is known to interact with several resident mitochondrial proteins, in particular with the voltage-dependent anion channel (VDAC) in the outer and with the adenine nucleotide translocase (ANT) in the inner mitochondrial membrane. Importantly, these proteins form the backbone of MPTP (McEnery et al., 1992; Gavish et al., 1999) and take part in the regulation of MPTP and apoptosis (Chelli et al., 2001, 2004; Azarashvili et al., 2007; Li et al., 2007). Moreover, PBR ligands can modulate oxidative stress (Fennell et al., 2001; Jayakumar et al., 2002) and PBR expression is increased in response to nervous tissue injury (Lacor et al., 1996, 1999; Weissman and Raveh, 2003). PBR is also thought to play a role in steroid biosynthesis and cholesterol transport, as well as in the control of mitochondrial energy production (for reviews, see Casellas et al., 2002; Papadopoulos et al., 2006a,b; Gavish et al., 1999).

Vinpocetine (14-ethoxycarbonyl-(3a,16a-ethyl)-14,15-eburnamine; Cavinton), a synthetic derivative of the lesser periwinkle plant (*Vinca minor*) alkaloid vincamine, is widely used as a neuroprotective agent for the prevention and treatment of central nervous system disorders of cerebrovascular origin. Its beneficial clinical effects are based on a wide range of action mechanisms including antioxidant, vasodilating and neuroprotective effects, however, the precise nature of the underlying mechanistic processes is not yet clear (Bonoczk et al., 2000; Vas and Gulyas, 2005). Recent studies indicated that Vinpocetine has a relatively high potency towards PBR, raising the possibility that the pharmacological action of Vinpocetine may involve the regulation of PBR function (Gulyas et al., 2005).

In the present study, we examined the neuroprotective effects of Vinpocetine in glutamate excitotoxicity assays on embryonic cortical neurons by analysing changes in mitochondrial membrane potential and cell viability. Since Vinpocetine has a binding affinity to PBR, neuroprotective effects of Vinpocetine were also compared to the effects of isoquinoline carboxamide (PK11195) and 4'-chlorodiazepam (Ro5-4864), two drugs with selective and high affinity to PBR (Le Fur et al., 1983).

2. Materials and methods

2.1. Primary neuronal cultures

Primary cortical neurons were prepared from 15- to 16-day-old mouse embryos. Under aseptic conditions, embryonic brains were prepared and transferred to DMEM supplemented with Amphotericin B (2.5 µg/ml) and 1% PenStrep (all from Sigma, NL). Cortices were isolated, cleaned from meninges and were incubated in 0.05% trypsin solution (Gibco, Invitrogen, NL) for 15 min at 37 °C. After a brief centrifugation, cells were triturated in NeuroBasal media supplemented with B27 (Gibco, Invitrogen, NL), 0.5 mM Glutamine, 1% PenStrep, 2.5 µg/ml Amphotericin B and filtered through a sterile polyester mesh with 42 µm pore size (EmTek Ltd., Hungary). Cell number was determined by trypan blue exclusion, and cells were seeded onto poly-D-lysine (PDL, Sigma, NL) coated Petri dishes or coverslips at 10^5 cells/cm² density in NeuroBasal media supplemented with B27, 0.5 mM Glutamine, 1% PenStrep, 2.5 µg/ml Amphotericin B. Cells were cultivated at 37 °C in 5% CO₂/95% air atmosphere. To prevent the division of non-neuronal cells, 2-day-old cultures were treated with 10 µM cytosine-arabino-furanoside (CAR, Sigma, NL) for 1 day.

2.2. Measurement of intracellular free calcium ($[Ca^{2+}]_i$) levels in single cells

Cortical neurons isolated from 15- to 16-day-old embryos were grown on PDL-coated glass coverslips in 35 mm dishes. 6 days after plating, cultures were washed with physiological solution containing 140 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl₂, 10 mM Hepes, 10 mM D-glucose, 10 µM glycine (pH 7.2, 300 mOsm) and loaded with 5 µM Fura-2AM dye (Molecular Probes, Invitrogen, Hungary) for 30 min at RT in the above buffer solution. At the end of the incubation, excess Fura-2AM was removed and cultures were further incubated for 30 min to completely hydrolyse the Fura-ester. $[Ca^{2+}]_i$ was determined under constant perfusion at RT (22 °C), using an inverted Olympus IX81 microscope with a 40× objective, MT-20 external illumination and the Cell^R software (Olympus, Hungary). Optical measurements were obtained by alternate excitation at 340 and 380 nm, with 350 ms frame rates. Fluorescent signals were detected by a cooled C9100-13 EM-CCD Hamamatsu camera (Hamamatsu Photonics, Herrsching am Ammersee, Germany), using a 510/30 nm bandpass filter. 25 µM glutamate (Sigma, Hungary) and 25 µM Vinpocetine (Richter Ltd., Hungary) were added via the perfusion system at 1 ml/min flow rate. 25 µM Vinpocetine was perfused for 30 min before the glutamate treatment and was also present during the 30 min long glutamate administration. Data were evaluated by the Cell^R software provided by Olympus (Hungary). In order to compare $[Ca^{2+}]_i$ responses of cells in different measurements, calculated $\Delta F/F$ values (changes in the ratio) were normalized to the highest value of the initial $[Ca^{2+}]_i$ response (100%). Normalized $\Delta F/F$ values were compared by Student's *t*-test (significance level was set to $p < 0.05$).

2.3. Drug preparation

Vinpocetine (Richter Ltd., Hungary), PK11195 (1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinolinecarboxamide, Sigma, NL) and Ro5-4864 (7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one, Sigma, NL) were stored at 4 °C in 10 mM stock concentration in cc. DMSO (Sigma, NL). FCCP (Mesoxalonnitrile 4-trifluoromethoxyphenylhydrazine, Sigma, Hungary) was dissolved in 95% ethanol to create 10 mM stock solution.

2.4. Glutamate excitotoxicity assays and viability tests

Glutamate excitotoxicity assays were performed on embryonic cortical neurons 6 days after plating. Conditioned media of the cultures were collected before the drug treatment. Cells were treated with different drugs at the indicated concentrations in fresh media for 30 min, followed by a 1 h long treatment with 33 µM glutamate. Drugs were present during the glutamate administration, as well. After the glutamate treatment, conditioned media were given back to the cells. Viability of the cultures was measured 24 h later by MTT assay, according to Mosmann (1983). Briefly, cells grown in 96-well plates were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) in a final concentration of 250 µg/ml. After 2 h incubation, cells and formazan crystals were dissolved in acidic (0.08 M HCl) isopropanol (Merck). Optical density was determined at a measuring wavelength of 570 nm against 630 nm as reference with Biorad ELISA reader. Assays were carried out on eight parallel wells. To compare the data obtained from different measurements, average viability values were expressed as a percentage of maximum cell death observed in cultures treated with glutamate only. Data are shown as averages and standard deviations and were compared by Student's *t*-test ($p < 0.05$).

2.5. Detection of mitochondrial membrane depolarisation

Analysis of mitochondrial membrane potential of primary neurons was performed by Becton-Dickinson FACSCalibur flow cytometer equipped with argon-ion laser according to the methods described by Lecoeur et al. (2004) with

modifications. Primary cortical neurons were plated onto 24-well plates at a density of 5×10^5 cells/well. 6 days after plating, neurons were treated with glutamate and drugs according to the experiments. During the last 20 min of glutamate treatment, cultures were stained by 40 nM 3,3'-diethyloxycarbocyanine iodide (DiOC₆(3), Molecular Probes/Invitrogen), a MMP-sensitive dye, at 37 °C in dark. At the end of the treatment, cells were washed by phosphate buffered saline (PBS) and incubated with 250 μ l/well of 37 °C 0.05% trypsin–EDTA solution (Gibco, Invitrogen) for 5 min at 37 °C. Cells were carefully harvested and suspended in 500 μ l B27 free NeuroBasal media (Gibco, Invitrogen) by gentle flushes. To label the dying cells, cell suspensions were stained by 10 μ g/ml propidium iodide (PI) (Sigma). Flow cytometry measurements were carried out immediately after trypsinisation.

Emission of DiOC₆(3) was detected in the FL1 channel (530/30 nm bandpass filter) and impaired cells were identified by their decreased MMP (DiOC₆(3)^{LOW} cells). Late apoptotic/necrotic cells were identified by increased propidium iodide uptake measured in the FL3 channel (670 nm longpass filter). We also used the forward side scatter (FSC) intensity, which roughly equals to the particle's size and can also be used to distinguish between cellular debris and living cells (see Fig. 4a). During the analyses, the FSC^{HIGH} and PI^{LOW} population was regarded as healthy cells. Data acquisition and analysis were performed using CellQuest Pro software. 20,000 cells were sorted in each measurement and only the FSC^{HIGH}/PI^{LOW} cell population (~55–60% of analysed cells) were used for detailed analyses. In order to compare the effects of the applied drugs, changes in the ratio of DiOC₆(3)^{LOW} cells upon different treatments were normalized to the change of DiOC₆(3)^{LOW} cells population caused by 150 μ M glutamate treatment alone (100%). Data are presented as averages and standard deviation from 3–3 different measurements of 10 independent cultures.

3. Results

3.1. Vinpocetine can reduce $[Ca^{2+}]_i$ elevations evoked by glutamate treatment

Former studies indicated that under excitotoxic conditions caused by opening of voltage-dependent sodium channels, Vinpocetine exerted neuroprotective effects at least partly by decreasing Ca^{2+} influx and intracellular free Ca^{2+} level, as well (Tretter and Adam-Vizi, 1998; Sitges and Nekrasov, 1999; Zelles et al., 2001; Sitges et al., 2006). To test whether Vinpocetine can activate similar mechanisms during glutamate-induced excitotoxicity, ratiometric Fura-2 Ca^{2+} imaging was performed in embryonal cortical neuronal cultures 6 days after plating (Fig. 1). Changes in $[Ca^{2+}]_i$ were measured upon exposure to 25 μ M glutamate, either without or in the presence of 25 μ M Vinpocetine (Fig. 1a). In case of Vinpocetine-treated cultures, Vinpocetine was perfused for 30 min already before the onset of glutamate treatment.

Regardless to the presence of Vinpocetine, initial $[Ca^{2+}]_i$ rise was similar in the neurons upon glutamate exposure (Fig. 1b). Within 10 min, however, elevated $[Ca^{2+}]_i$ level was reduced significantly (Fig. 1c), which was maintained throughout the additional 20 min of the investigations, as well (81.7 ± 10.8 or $109.2 \pm 9.1\%$ of the initial $[Ca^{2+}]_i$ peak in cultures with or without Vinpocetine treatment, respectively. $p < 0.001$).

3.2. Neuroprotective effect of Vinpocetine and peripheral benzodiazepine receptor-binding compounds after glutamate excitotoxicity

According to recent studies, Vinpocetine has binding affinity to peripheral benzodiazepine receptor (Gulyas et al., 2005; Vas et al., 2008). Vinpocetine-mediated effects on embryonal cortical neurons were measured in toxicity tests and compared to the action of isoquinoline carboxamide (PK11195) and 4'-chlorodiazepam (Ro5-4864), which have selective and high binding affinity to PBRs (Gavish et al., 1999; Casellas et al., 2002). Based on the entropy-driven and enthalpy-driven nature of ligand–receptor interactions, PK11195 and Ro5-4864 have been classified as an antagonist and agonist, respectively (Le Fur et al., 1983).

Neuroprotective action of 1.5 h treatment with Vinpocetine, PK11195 or Ro5-4864 was determined by MTT assay, 24 h after

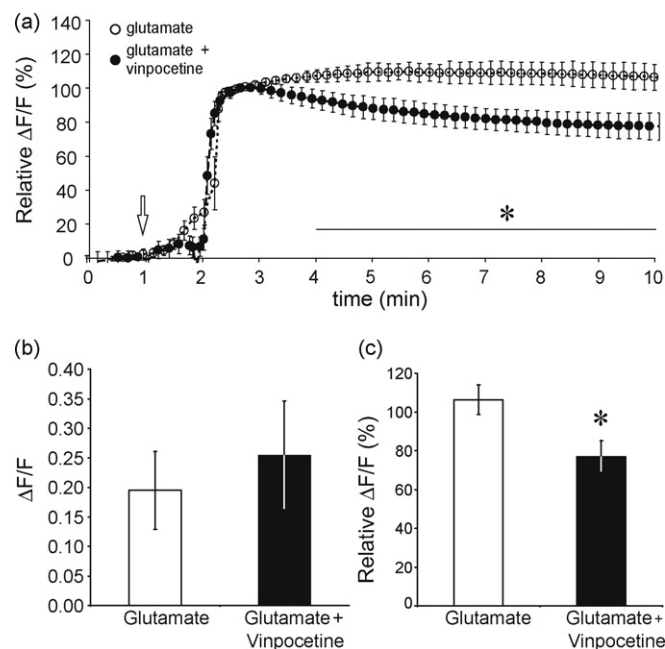


Fig. 1. Intracellular Ca^{2+} changes in embryonic cortical neurons during 25 μ M glutamate treatment in the presence or in the absence of 25 μ M Vinpocetine. (a) Average relative $\Delta F/F$ values of glutamate (open circles; $n = 55$) or glutamate and 25 μ M Vinpocetine (black circles; $n = 31$) treated neurons. $\Delta F/F$ values were normalized to the highest value of the initial $[Ca^{2+}]_i$ response (100%). Arrow indicates the start of 25 μ M glutamate administration, which was continued for 30 min. Data are presented as average and standard deviation; * $p < 0.05$. (b) Peak $\Delta F/F$ values of the initial Ca^{2+} answers of neurons treated by glutamate (white bar) or glutamate and Vinpocetine (black bar). (c) Relative $\Delta F/F$ values determined 9 min after the onset of glutamate or glutamate and Vinpocetine treatment. Data are presented as average and standard deviation and are related to the level of the initial $[Ca^{2+}]_i$ rises (100%); * $p < 0.001$.

the onset of drug administration. According to our data, neither of the compounds had any toxic effects in the 10 nM to 50 μ M concentration range (data not shown). In glutamate excitotoxicity assays initiated by 1 h treatment with 33 μ M glutamate, 1 μ M Vinpocetine already exerted slight neuroprotection, which was increased further in a dose-dependent manner (Fig. 2). 50 μ M Vinpocetine had a strong neuroprotective effect and decreased cell death by $74.9 \pm 7.6\%$. On the other hand, Ro5-4864 and PK11195 exerted significantly lower neuroprotection and were effective only in high (>25 μ M) concentration. In case of 50 μ M treatment, Ro5-4864 had a significantly increased neuroprotective action compared to PK11195 (76.9 ± 5 and $65.5 \pm 8\%$, respectively; $p < 0.001$).

The level of neuroprotection upon combining Vinpocetine with either PK11195 or Ro5-4864 was also investigated (see Fig. 3a for the experimental schedule). At the beginning of the experiments, PK11195 or Ro5-4864 was added to the cultures in various concentrations for 30 min followed by an additional 10 μ M Vinpocetine treatment. After a further 30 min of incubation, 33 μ M glutamate treatment was applied for 1 h in the presence of all previously added compounds. 24 h upon the removal of glutamate and the various drugs from the cultures, cell viability was checked by MTT assay. Both PK11195 (Fig. 3b) and Ro5-4864 (Fig. 3c) increased neuroprotection exerted by Vinpocetine alone (indicated by dashed lines in Fig. 3b and c) in a dose-dependent manner, but significant alterations were found only when PK11195 and Ro5-4864 were present at a concentration higher than 10 μ M. These findings already indicate that the mode of action exerted by Vinpocetine and the PBR-binding drugs may be different.

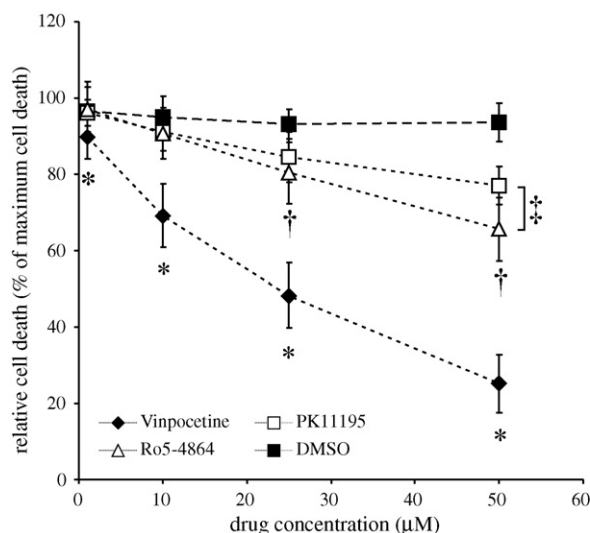


Fig. 2. Dose-dependent neuroprotective effects of 10–50 μM Vinpocetine (filled diamond), Ro5-4864 (open triangle) or PK11195 (open box) on cortical neurons. Control cultures were treated with DMSO only (filled box). In all cases, cells were pretreated with the corresponding drugs for 30 min before the onset of the glutamate treatment (1 h, 33 μM). Drugs were present also during glutamate administration. Cellular viability was measured by MTT assay 24 h after the glutamate treatment and data were normalized to the level of maximum cell death observed in cultures treated only with glutamate. Data are presented as average and standard deviation. * $p < 0.01$, comparing Vinpocetine-treated values to solvent-only treated values; $^{\dagger}p < 0.01$, comparing Ro5-4864 and PK11195 treated values to solvent-only treated values; $^{\ddagger}p < 0.01$, comparing Ro5-4864 and PK11195 treated values to each other.

3.3. Vinpocetine can attenuate mitochondrial membrane depolarisation caused by excitotoxic conditions

PBRs are suggested as members or modulators of the MPTP (McEnery et al., 1992; Casellas et al., 2002; Papadopoulos et al., 2006a), whose opening can directly influence MMP (Kroemer and Reed, 2000). According to previous publications, Vinpocetine has a binding affinity towards PBRs (Gulyas et al., 2005; Vas et al., 2008), raising the possibility that Vinpocetine can affect mitochondrial membrane potential changes via interacting with PBRs. In order to test this hypothesis, we used an MMP-sensitive dye, 3,3'-dihexyloxacarbocyanine iodide in flow cytometry to follow changes in neuronal MMP upon glutamate treatment.

As deregulation of MMP upon excitotoxic stimuli precedes apoptotic processes and cell death (Nicholls and Ward, 2000; Nicholls, 2004), flow cytometry was carried out directly after the 1 h long treatment with glutamate. In this case, treatment with 25 μM Vinpocetine, Ro5-4864 or PK11195 was started 30 min before the glutamate exposure and continued during the glutamate administration period. After the incubation steps, primary cortical neurons were gently harvested by trypsinisation and cell suspensions were subjected to flow cytometry. Membrane integrity of the harvested neurons was checked by propidium iodide staining, cell volume was characterised by forward side scatter value and only healthy cells were used for evaluations (PI^{LOW} and FSC^{HIGH} cells; Fig. 4a). Addition of 10 μM FCCP, a known mitochondrial protonophor (Azarashvili et al., 2007), strongly shifted $\text{DiOC}_6(3)$ fluorescence towards lower values, while 25 mM KCl treatment, known to depolarise plasma membrane, did not have lower MMP (Fig. 4b). Initial titration experiments also showed that treatment with 150 μM glutamate significantly increased the ratio of cells possessing low MMP ($\text{DiOC}_6(3)^{\text{LOW}}$ population) (Fig. 4c). These data unequivocally show that our method was suitable to compare the effects of Vinpocetine to effects of the PBR ligands upon glutamate-induced MMP changes.

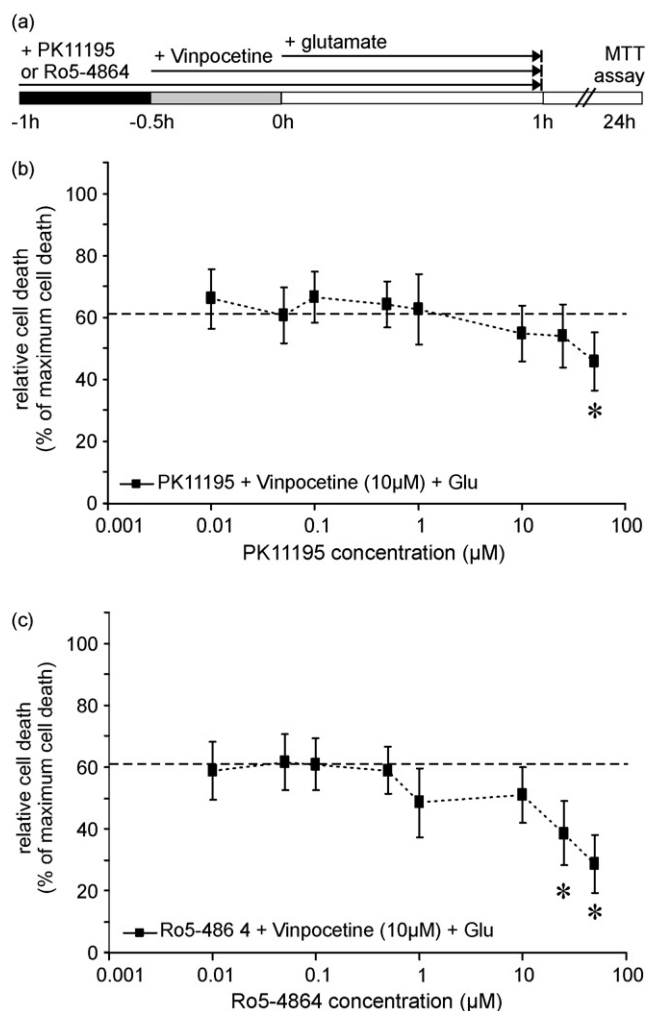


Fig. 3. Neuroprotective effects of Vinpocetine combined with PBR-selective drugs. (a) Schematic representation of the experimental design. Neurons were treated with PK11195 (b) or Ro5-4864 (c) in various concentrations for 30 min, then 10 μM Vinpocetine was also added to the cultures. Excitotoxicity was induced by 1 h of glutamate treatment (33 μM) and cellular viability was measured 24 h after the glutamate treatment. Data were normalized to the level of maximum cell death observed in control, glutamate-only treated cultures (100%). On (b) and (c), dashed line indicates the average neuroprotection evoked by 10 μM Vinpocetine alone. Data are presented as averages and standard deviations; * $p < 0.05$, in comparison to the Vinpocetine-only treated values.

When Vinpocetine, Ro5-4864 or PK11195 (all in 25 μM) were present during glutamate treatment, the amount of cells with decreased MMP was significantly decreased compared to glutamate-only treated cultures (Fig. 4d). On the other hand, Ro5-4864 was significantly more effective than Vinpocetine or PK11195 treatment alone, while PK11195 exerted similar protective effects to Vinpocetine (Table 1). Moreover, the combination of Vinpocetine with Ro5-4864 or PK11195 further decreased the proportion of $\text{DiOC}_6(3)^{\text{LOW}}$ population indicating a stronger neuroprotection. The changes were significant in both cases (Table 1). According to these results, strongest neuroprotection was obtained with the combined use of Vinpocetine together with Ro5-4864.

4. Discussion

Under *in vivo* ischemic conditions, primary neuronal cell death appears rapidly in the ischemic core region. As a result of the activation of multiple death pathways, this initial process is

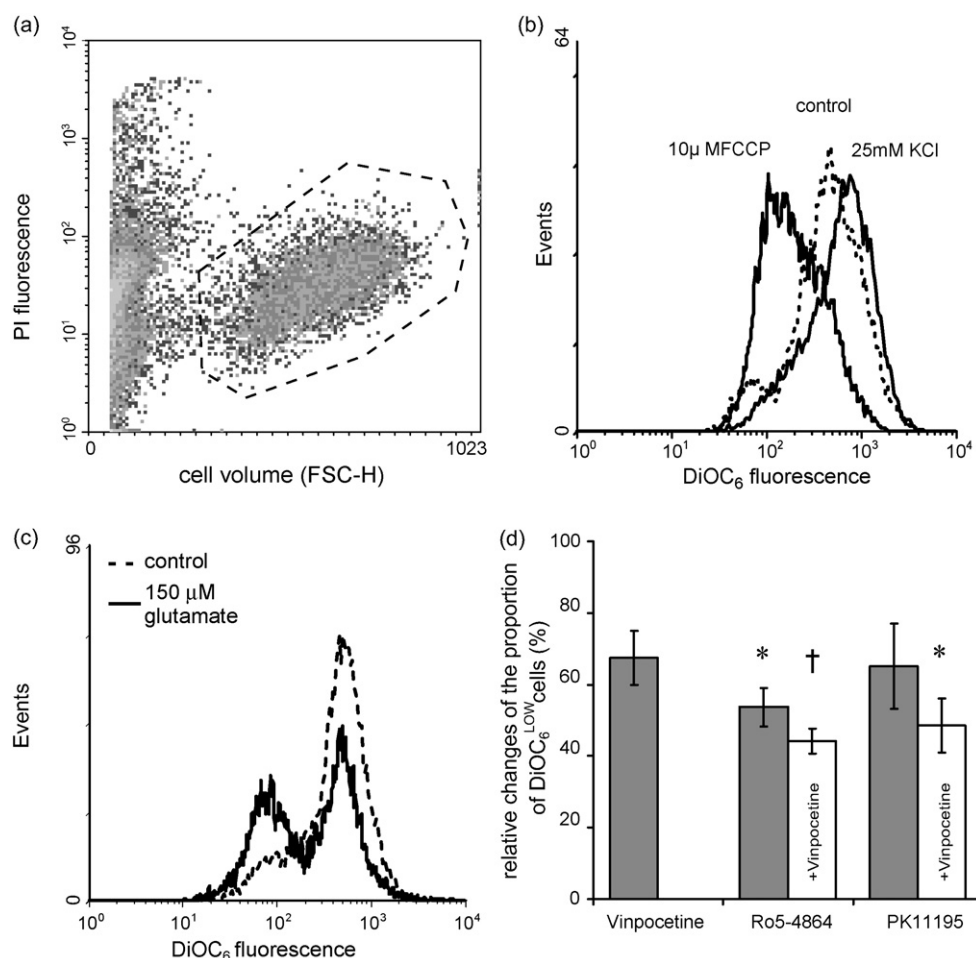


Fig. 4. Effects of Vinpocetine, Ro5-4864 and PK11195 on mitochondrial membrane potential (MMP) during glutamate treatment. MMP of dissociated cortical neurons was measured by flow cytometry using DiOC₆(3), a MMP-sensitive dye. (a) For data evaluation, only the healthy (FSC^{HIGH} and PI^{LOW}) cells were considered (see cells plotted in the area delineated by the dashed line). (b) 10 μM FCCP strongly shifted DiOC₆(3) fluorescence towards lower MMP values, while 25 mM KCl treatment slightly enhanced DiOC₆(3) labelling. (c) 150 μM glutamate (black line) increased the amount of cells with low DiOC₆(3) fluorescence. (d) Relative changes in the proportion of DiOC₆(3)^{LOW} cells after glutamate treatment and in combination with Vinpocetine, Ro5-4864 or PK11195 treatment. Data were normalized to the change of DiOC₆(3)^{LOW} cells population caused by 150 μM glutamate treatment alone (100%). The ratio of neurons possessing low MMP in glutamate and various drug-treated cultures was decreased significantly compared to the effects of glutamate treatment alone (not indicated; $p < 0.001$). Ro5-4864 in itself exerted higher neuroprotection than Vinpocetine alone ($*p < 0.01$). The combination of Vinpocetine with Ro5-4864 or PK11195 increased further the neuroprotective effect ($†p < 0.05$, in comparison to the Vinpocetine-only treated values, for more details, see Table 1).

followed by a secondary delayed cell death in the penumbra area (see Won et al., 2002 for a review). A key element of the secondary cell loss is the increased activity of excitatory amino acid regulated receptors, which elevates $[Ca^{2+}]_i$ above the physiological levels and leads to disturbed functions and eventually cell death (De Keyser et al., 1999; Nicholls, 2004; Hazell, 2007). Former clinical and experimental studies *in vivo* and *in vitro* have shown that Vinpocetine, developed by Richter Ltd., is able to protect the neurons under ischemic condition by a variety of actions (Bonoczk

et al., 2000; Vas and Gulyas, 2005). Vinpocetine can block phosphodiesterase I, which can be important for improvement of cerebral circulation (Pelligrino and Wang, 1998) and can act as an antioxidant (Vas and Gulyas, 2005). Vinpocetine also blocks voltage-gated sodium channels (VDSCs) in therapeutically relevant concentrations ($<50 \mu\text{M}$), decreases the Ca^{2+} influx evoked by VDSC stimulation and can also attenuate Ca^{2+} -dependent destructive processes (Lakics et al., 1995; Tretter and Adam-Vizi, 1998; Sitges and Nekrassov, 1999; Zelles et al., 2001; Sitges et al., 2006).

Table 1

Effects of Vinpocetine, Ro5-4864 and PK11195 on mitochondrial membrane potential (MMP) during glutamate treatment

Treatment	Relative changes in the proportion of DiOC ₆ ^{LOW} cells (%)	<i>p</i> values compared to Vinpocetine-only treated cultures	<i>p</i> values compared to Ro5-4864- or PK11195-only treated cultures
Vinpocetine	67.43 ± 7.43*		
Ro5-4864	53.74 ± 5.41*	0.0006	
Ro5-4864 and Vinpocetine	44.14 ± 3.47*	0.0002	0.024
PK11195	64.99 ± 11.90*	0.6066	
PK11195 and Vinpocetine	48.56 ± 7.45*	0.0021	0.068

Data were normalized to the change of DiOC₆(3)^{LOW} cells population caused by 150 μM glutamate treatment alone (100%). * indicates significant changes ($p < 0.0001$) compared to cultures treated with glutamate only. *p* values less than 0.05 were regarded as significant changes.

The present study shows that therapeutically relevant concentration ($<50 \mu\text{M}$) of Vinpocetine helps to recover increased $[\text{Ca}^{2+}]_i$ level evoked by chronic glutamate administration in primary cortical neuronal cultures. This result is consistent with former findings on veratridine-induced $[\text{Ca}^{2+}]_i$ changes (Tretter and Adam-Vizi, 1998; Zelles et al., 2001; Sitges et al., 2006). Importantly, Vinpocetine did not influence the amplitude of the initial glutamate-evoked $[\text{Ca}^{2+}]_i$ rise indicating that Vinpocetine acts at least partially by influencing mechanisms controlling elevated $[\text{Ca}^{2+}]_i$ levels. Recent positron emission tomography (PET) studies using radiolabeled PBR ligands and Vinpocetine suggested that mitochondrial peripheral-type benzodiazepine receptors can be therapeutically relevant targets of Vinpocetine (Gulyas et al., 2005; Vas and Gulyas, 2005). As mitochondria play an important role in Ca^{2+} homeostasis, neuroprotective effects of Vinpocetine can be executed through interfering with PBRs and consequently, via modulating mitochondrial function.

In the last decade, the involvement of PBRs in neurological conditions was extensively investigated using PBR ligands or PBR overexpressing cells (Gavish et al., 1999; Fischer et al., 2001; Casellas et al., 2002; Papadopoulos et al., 2006b). Accordingly, PBRs were found to be preferentially localised in the outer membrane of mitochondria (O'Beirne and Williams, 1988; Hirsch et al., 1989). Several studies indicated that PBR-mediated changes in mitochondrial function can play a key role during apoptotic events (Tanimoto et al., 1999; Casellas et al., 2002; Chelli et al., 2004; Azarashvili et al., 2005; Li et al., 2007). Recently, PBRs have been shown to directly regulate MPTP opening (Chelli et al., 2001; Azarashvili et al., 2007), which is one of the first steps in apoptotic cascades.

To investigate the role of PBRs in glutamate excitotoxicity, we compared the neuroprotective effect of Vinpocetine with the beneficial action of the specific synthetic PBR ligands, isoquinoline carboxamide (PK11195) and 4'-chlorodiazepam (Ro5-4864). In our *in vitro* glutamate excitotoxicity assays, PK11195 or Ro5-4864 exerted significantly less neuroprotection and needed higher concentration than that observed in Vinpocetine-treated cultures. Combination of the different drug treatments increased neuroprotection, indicating partly different mode of action between Vinpocetine and PK11195 or Ro5-4864. These results suggest that the underlying mechanisms and/or the binding sites of Vinpocetine versus PK11195 or Ro5-4864 may be at least partially different, which was also suggested in PET studies, as well (Vas et al., 2008).

The neuroprotective role of PK11195 and Ro5-4864 is a matter of debate and both pro- and anti-apoptotic effects in neuronal primary cultures have been reported (Jayakumar et al., 2002; Parker et al., 2002; Jorda et al., 2005; Mills et al., 2005). In our hands, Ro5-4864 proved to be more potent than PK11195 to protect the cells from glutamate-evoked excitotoxicity both in viability tests and flow cytometry experiments. This is in line with former observation on hippocampal neurons during kainic acid-induced excitotoxicity (Veiga et al., 2005) or in sensory neuron regeneration (Mills et al., 2005). The different action of these drugs may rely on their different binding site(s) or on the different modulation of their binding sites by other members of the PBR complex, e.g. by VDAC (Joseph-Liauzun et al., 1997; Lacapere et al., 2001; Garnier et al., 1994).

Interestingly, Ro5-4864 proved to be more effective in preserving normal MMP than Vinpocetine alone. Furthermore, the effects of PBR drugs were potentiated by Vinpocetine. These findings suggest that regulation of $[\text{Ca}^{2+}]_i$ levels and MMP can take place in partially different ways and allow the conclusions that PK11195 and Ro5-4864 exert a more direct action on mitochondria than Vinpocetine.

Taken together, our results suggest that during *in vitro* glutamate excitotoxicity, neuronal PBRs are not likely the primary targets for the neuroprotective action exerted by Vinpocetine. The outcome of combined treatment with Vinpocetine and specific PBR associated drugs in excitotoxicity experiments and mitochondrial membrane potential preservation indicates at least a partly different mode of action. It is a feasible explanation that the observed restoration of MMP and the reduction of excitotoxic cell death is a consequence of Vinpocetine's attenuating effect on elevated intracellular Ca^{2+} levels.

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